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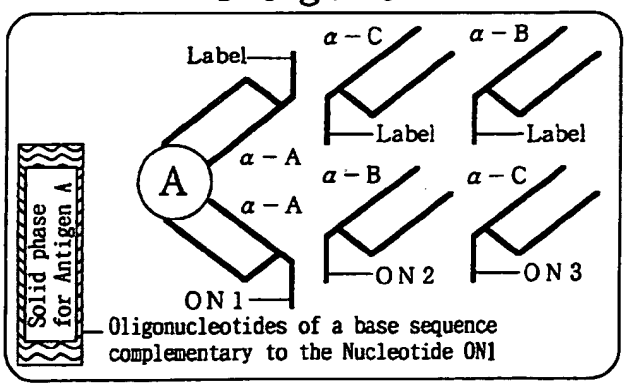
(54) METHOD FOR ASSAYING MORE THAN ONE IMMUNOLOGICAL LIGAND, AND ASSAY REAGENT AND KIT THEREFOR

(57) An assay reagent and a kit whereby nonspecific capture of a labeled substance by a solid phase can be suppressed and more than one antibody or more than one antigen can be detected via a simple assay by using one reagent. An immunological ligand assay reagent comprising components selected from the following groups (A) and (B) at the same time is reacted with immunogical ligands to thereby form complexes, which are captured by separate solid phases (C) and analyzed based on the labeling substance contained in each complex: group (A): more than one type of immunological antiligand-nucleotide conjugates wherein antibodies having specific avidities respectively for immunological ligands of different types are bonded to nucleotides which are specific respectively for said immunological ligands and each has an arbitrarily selected base sequence; group (B): labeled substances having specific avidities respectively for the immunological antiligands of different types which are to be assayed; and group (C): solid phase-nucleotide conjugates wherein nucleotides having base sequences capable of complementarily binding respectively to the nucleotides of the group (A) are bonded to a water-insoluble carrier. Either antigens or antibodies are applicable to said immunological ligands and immunological antiligands.

Fig. 5



EP 0 698 792 A1

Description

TECHNICAL FIELD

The present invention relates to a method for immunologically assaying biological substances using an antigen-antibody reaction. More specifically, the present invention relates to a method for as saying one or more species of antigens or one or more species of antibodies, characterized in that the method is capable of assaying an almost infinite number of the combination of one or more species of antigens or one or more species of antibodies, an assay reagent using the same and a kit using the same.

BACKGROUND ART

Immunoassays have been used in the field of clinical diagnosis for assaying and detecting a trace of biological substances, and a variety of methods have been developed therefor. Because immunoassays use non-radioactive substances such as fluorescent substances, luminescent substances and enzymes as labeling substances of antibodies, and therefore, do not require specific equipment as is required when a radioactive substance is used as a labeling substance, such assays have been more widely used among methods for assaying biological substances, from the respect of the easy handling of the reagents and the processability of a great number of samples.

By such immunoassays, generally, only a single antigen may be assayed or detected in a single clinical sample. When a plurality of antigen species are present in a sample which are defined as Antigens A, B and C, for example, a specific reagent for selectively detecting the Antigen A should be required, which is also the case with the Antigens B and C. Thus, these antigens also require their respective specific reagents. Generally, a number of clinical test results are integrally required for clinically diagnosing the disease of a patient. So as to receive appropriate treatment under appropriate diagnosis, accordingly, such patient generally should be subjected to a plurality of clinical tests. For that reason, in most cases, the volume of a sample collected from a patient increases in proportion to the number of clinical tests required for that patient, which is a bodily burden for the patient. For the demand to decrease such burden, no satisfactory assay method, simple and highly sensitive, is present currently.

As an immunoassay method to simultaneously determine the presence and/or level of one or more species of antibodies in a sample, dot blotting has conventionally been known for example from Japanese Patent Laid-Open No. Hei 4-232465 (1992). Dot blotting comprises preliminarily spotting various species of antigens on a nitrocellulose membrane, reacting a sample possibly containing a plurality of antibodies to be detected with the antigens on the nitrocellulose membrane, and subsequently binding a labeling substance to the antibodies captured on the nitrocellulose membrane to detect the presence or level of the antibodies. However, such dot blotting for detecting one or more species of antibodies is problematic in that the entire process thereof requires a long time and also requires a larger volume of a sample. Furthermore, the dot blotting has a problem in that since antigens should be immobilized onto a solid phase for use in assaying antibodies, the immobilized antigens are deteriorated under storage, disadvantageously.

Alternatively, Fig. 1 depicts the scheme of the general process of another immunoassay method different from the dot blotting, which is called sandwich assay as one of conventional immunoassay methods by means of a labeled compound. Conventional sandwich assays by means of a labeling compound will now be described with reference to Fig. 1, wherein 1 represents solid phase of a water-insoluble support; 2 represents antibody immobilized onto the solid phase; 3 represents an antigen to be assayed, which has reacted with the antibody and then bound to the antibody; and 4 represents a labeled antibody.

Bringing firstly a testing sample in contact with antibody 2 immobilized onto solid phase 1, the antigen to be detected in the testing sample should be bound to the antibody 2 (Fig. 1(a)) So as to remove the unreactive contaminants in the reaction system, washing is carried out (Fig. 1(b)). Labeled antibody 4 reacts with the solid phase-antibody-antigen complex, to form a solid phase-antibody-antigen-labeled antibody complex (Fig. 1(c)). Washing is carried out so as to remove the contaminants such as unreactive labeled antibody and the like in the reaction system after the above process (Fig. 1(d)). By detecting the label, the washed solid phase-antibody-antigen-labeled antibody complex is determined (Fig. 1(e)).

By such general immunoassay methods, the labeled antibody responsible for the determination is derived from the solid phase-antibody-antigen-labeled antibody complex. Additionally, so-called non-specific binding of the labeled antibody directly onto the solid phase is sometimes incurred, and in such case, the assay sensitivity is eventually decreased disadvantageously.

Many of pathogenic factors such as specific hormones, tumor markers and bacteria are present at a trace level in samples. For the assay or detection then, a highly sensitive assay system has been required and developed (see for example Enzyme immunoassay, Eiji Ishikawa eds., Igaku Shoin, 1987; Microbiol. Immunol., Y. Oku et al, 32, pp. 807-816, 1988). The major problem in constructing the highly sensitive assay system is such non-specific binding of the labeled antibody onto the solid phase.

For the purpose of eliminating such non-specific binding, immune complex transfer assay (J. Biochem., S. Hashida et al., 108, pp. 960-964, 1990) and the like, have been developed to attain practically higher sensitivity than those of conventional assays. The immune complex transfer assay comprises the following steps. More specifically, the assay comprises simultaneously mixing an antibody conjugated with a dinitrophenyl group (referred to as "DNP" group) and biotin, the antibody recognizing a specific antigen, a testing sample, and an enzyme-labeled antibody recognizing the antigen, to facilitate the reaction of the antigen contained in the testing sample with the individual antibodies to form an immune complex (referred to as "IC" hereinafter). Alternatively, immobilizing an antibody against DNP group onto a solid phase, bringing the preliminarily prepared IC in contact with the solid phase to promote the antigen-antibody reaction between the DNP group contained in the IC and the antibody against DNP group on the solid phase, the IC is captured onto the solid phase.

Then, so as to exclude the effects of the non-specific binding of the enzyme-labeled antibody contained in the reaction system on the solid phase, the captured IC is released therefrom through the addition of an excess amount of a compound with a DNP group. Subsequently, the thus released IC is subjected to the reaction with another solid phase immobilizing avidin, to capture again the IC onto the solid phase, and then, the activity of the enzyme in the IC captured onto the solid phase is measured in a test tube, whereby the effects of the non-specific binding of the enzyme-labeled antibody on the solid phase can be eliminated, to attain high sensitivity. However, none of such conventionally known immune complex transfer assays indicates the detection and assay of the presence of one or more species of antigens or one or more species of antibodies by means of a single reagent.

Furthermore, Japanese Patent Laid-Open No. Sho 63-188399 (1988) describes a method for assaying a target molecule as a biological binding pair in a sample. Specifically, the publication describes that the assay procedure comprises bringing a sample containing a target molecule in contact with a first anti-ligand probe and a second labeled anti-ligand probe capable of bonding the target molecule to form a complex and a recoverable support, then substantially separating the recoverable support from the sample to recover an isolated product including the target molecule and the first and second probes in the presence of the target molecule in the sample, and further assaying the presence of the target product indicating the presence of the target molecule. However, the assay procedure described in the Japanese Patent Laid-Open No. Sho 63-188399 never describes the detection and assay of the presence of one or more species of antigens or one or more species of antibodies by means of a single reagent.

Additionally, Japanese Patent Laid-Open No. Hei 4-273065 (1992) describes a method for detecting an antigen contained in a sample at a high sensitivity, comprising preliminarily immobilizing an antibody through a nucleic acid onto a solid phase, capturing an antigen contained in a sample via the antigen-antibody reaction onto the solid phase, thereafter capturing a labeling substance thereon prior to washing, selectively cleaving the nucleic acid to separate and assay the separated labeling substance. However, the method described in the Japanese Patent Laid-Open No. Hei 4-273065 never describes or indicates anything about the detection and assay of the presence of one or more species of antigens or one or more species of antibodies via a single reagent.

As has been described above, the entire process of the conventional dot blotting for detecting one or more species of antibodies has required a long time and additionally requires a relatively larger volume of a sample.

Because the reaction for capturing the labeled antibody onto the solid phase is an antigen-antibody reaction in the conventional immunoassay method depicted in Fig. 1, the time required for capturing the labeled antibody onto the solid phase is relatively long in unit of hour. Thus, the duration of the labeling substance of the reaction system being exposed to the solid phase is so much prolonged that so-called non-specific binding of the labeling substance directly onto the solid phase is incurred, which is problematic because of the decrease in assay sensitivity.

Furthermore, the conventional immune complex transfer assay methods intended for higher sensitivity require a greater number of complex assay procedures as well as a long time for those reactions, which is the principal drawback.

It is thus a first objective of the present invention to suppress at minimum the occurrence of non-specific binding onto a solid phase of a labeling substance which should be added to a reaction system, which disadvantageously causes the decrease of assay sensitivity. In addition to the first objective, furthermore, it is a second objective of the present invention to provide an assay reagent which can detect one or more species of antibodies or one or more species of antigens by means of a single reagent by a simple procedure, a kit using the same and an assay method using the same.

DISCLOSURE OF THE INVENTION

So as to overcome the problems described above, the present invention is to provide an assay reagent for assaying one or more species of immunological ligands, which concurrently contains the following substance groups (A) and (B):

Substance group (A): one or more species of immunological anti-ligand-nucleotides conjugates, in each of which nucleotides with a specific base sequence, independently selected depending on the species of an immunological ligand, are bound to an immunological anti-ligand having a specific immunological affinity to one of immunological ligands as different species of subjective substances; and

Substance group (B): labeled substances each having a specific affinity to one of the immunological ligands as the different species of subjective substances.

Additionally, the present invention is an assay kit for assaying one or more species of immunological ligands, comprising an assay reagent for assaying one or more species of immunological ligands which concurrently contains the following substance groups (A) and (B), and the following solid phase (C):

Substance group (A): one or more species of immunological anti-ligand-nucleotides conjugates, in each of which nucleotides with a specific base sequence, independently selected depending on the species of an immunological ligand, are bound to an immunological anti-ligand having a specific immunological affinity to one of immunological ligands as different species of subjective substances;

Substance group (B): labeled substances each having a specific affinity to one of the immunological ligands as the different species of subjective substances; and

Solid phase (C); solid phase-nucleotides conjugate wherein nucleotides having a base sequence complementarily binding to the nucleotides of the above Substance group (A) are immobilized onto a water-insoluble support.

In accordance with the present invention, the term " immunological ligand" means one molecule in an immunologically formed pair, while the term "immunological anti-ligand" means the other molecule in the immunologically formed pair. These immunological ligand or immunological anti-ligand specifically means either one of antigen or antibody.

As the nucleotides having a complementary sequence in accordance with the present invention, use may be made of DNA or RNA. For such nucleotides, both of synthetic nucleotides and naturally occurring nucleotides may be used. The nucleotides may be an oligonucleotide and a polynucleotides.

An antibody-nucleotides conjugate or an antigen-nucleotides conjugate is bound to nucleotides immobilized onto a solid phase via the complementary paring of the base sequences of these nucleotides. Their complementary base sequences may be complementary, partially or wholly, as long as the nucleotide molecules thereof can bind to each other.

Generally, the complementary pairing of nucleotides characteristically has such a high specificity that the time required for such complementary pairing is far shorter than the time required for the formation of an antigen-antibody complex. In accordance with the present invention, the time required for binding an antibody-nucleotides conjugate or an antigen-nucleotides conjugate to a solid phase immobilizing other nucleotides (nucleotides-immobilized solid phase) is far shorter than the time required for binding a labeled substance to a complex of a solid phase immobilizing an antibody thereon and an antigen (an antibody-bound solid phase-antigen complex) in accordance with the conventional immunoassay. The reason is that by the conventional method for assaying an immunological ligand, namely an antigen or an antibody, a subjective substance conjugated to a labeled substance is captured onto a solid phase through an antigen-antibody reaction which requires longer time, but for the method for assaying immunological ligands in accordance with the present invention, a subjective substance conjugated to a labeled substance is captured onto a solid phase through the complementary pairing of nucleotides which advantageously requires far shorter time than the time required for antigen-antibody reaction.

Because a labeled substance, for example, a labeled antibody, cannot be given sufficient time for directly binding to a solid phase in accordance with the present invention, non-specific binding can be decreased to attain a highly sensitive assay system.

In accordance with the present invention, furthermore, only a single reagent is capable of detecting or assaying one or more species of immunological ligands, namely antigens or antibodies, so that the time required for their detection or assay is markedly shortened, compared with the conventional methods.

As to the solid phase-nucleotides conjugate as the Solid phase (C), the nucleotides may be covalently bonded at position 5' terminus or 3' terminus or an optional position other than the termini onto a water-insoluble support, directly or through a functional group inserted into the water-insoluble support, to form a solid phase-nucleotides conjugate as the Solid phase (C). A functional group thus inserted into a solid phase may be covalently bonded to a functional group inserted into a base constituting a nucleotide, to form a covalent bond.

As another method for binding nucleotides onto a solid phase, physical adsorption may be adopted instead of covalent bonding for such binding. More specifically, nucleotides are covalently bonded, at position 5' terminus or 3' terminus or an optional position other than such termini onto a bonding ligand, directly or through a functional group inserted into the bonding ligand, to form a bonding ligand-nucleotides conjugate, which is then physically adsorbed onto a water-insoluble support to form a solid phase-nucleotides conjugate as the Solid phase (C). Preferably, the bonding ligand may be a protein.

For the label to be inserted into a labeled substance in the assay reagent for assaying immunological ligands in accordance with the present invention, use may be made of an enzymatically active atomic group, biotin, avidin, digoxigenin, nucleotides, a metal colloid particle, a fluorescence substance, a luminescence substance, a metal compound, a ligand with a specific binding affinity, or a radioisotope. For the solid phase to be used in accordance with the present invention, preferably, use may be made of polystyrene for example.

The method for assaying one or more species of immunological ligands in accordance with the present invention will now be explained with reference to Figs. 2 to 10, wherein an antigen is illustrated as the immunological ligand to be assayed.

Fig. 2 depicts one example of the case wherein the assay reagent for assaying one or more species of immunological ligands is an assay reagent for assaying one or more species of antigens, schematically depicting the assay reagent for

assaying one or more species of antigens, Antigens A, B and C. In Fig. 2, those in frame all represent the components contained in a single reagent. In Fig. 2, α-A, α-B and α-C represent antibodies against Antigens A, B and C, respectively. The Antibodies α-A, α-B and α-C, each bound with a label, are thus prepared into modified labeled substances, so the reagent thus contains three types of labeled substances simultaneously. Concurrently with the aforementioned three types of the labeled substances, the reagent furthermore contains three types of antibody-nucleotides complexes composed of Antibodies α-A, α-B and α-C, which are independently bound with different sequences of Nucleotides, ON1, ON2 and ON3, in this order. The Nucleotide ON1 has a base sequence different from those of other Nucleotides ON2 and ON3. The Nucleotide ON2 has a base sequence different from those of other Nucleotides ON1 and ON3. The Nucleotide ON3 has a base sequence different from those of other Nucleotides ON1 and ON2. Nucleotides of an identical base sequence should be bound to an identical species of antibody. In accordance with the present invention, polyclonal antibodies are also encompassed in the term "an identical species of antibody."

Fig. 3 depicts one example of the solid phase attached with nucleotides, for use in combination with the assay reagent for assaying one or more species of antigens in accordance with the present invention; Fig. 3(a) depicts a solid phase attached with nucleotides (ON) of a base sequence complementary to the Nucleotide ON1 (Solid phase for Antigen A); Fig. 3(b) depicts a solid phase attached with nucleotides (ON) of a base sequence complementary to the Nucleotide ON2 (Solid phase for Antigen B); and Fig. 3(c) depicts a solid phase attached with nucleotides (ON) of a base sequence complementary to the Nucleotide ON3 (Solid phase for Antigen C). These different types of individual solid phases are independently and separately present. When used in combination with the assay reagent for assaying one or more species of antigens of Fig. 2, these individual solid phases constitute one example of the assay kit in accordance with the present invention.

When the assay reagent for assaying one or more species of antigens of Fig. 2 is added to a testing sample containing the Antigens A, B and C, complexes of (antibody-nucleotides conjugate)-antigen-labeled substance, individually corresponding to the Antigens A, B and C, may be formed in a reaction solution. The aspect is depicted in Fig. 4.

Then, bringing the reaction solution containing the complexes of (antibody-nucleotides conjugate)-antigen-labeled substance, in contact with the Solid phase for Antigen A, the Solid phase for Antigen B and the Solid phase for Antigen C, all of which phases are independently and separately present individually, the nucleotides contained in the complexes are complementarily paired through hybridization with the nucleotides of the Solid phases for Antigens A, B and C, having complementary base sequence to those of the nucleotides contained in the above complexes.

Figs. 5, 6 and 7 depict the complexes captured via nucleotide hybridization onto the individual solid phases; Fig. 5 depicts a complex carrying Antigen A, which is captured via nucleotide complementary pairing onto the Solid phase for Antigen A; Fig. 6 depicts a complex carrying Antigen B, which is captured via nucleotide complementary pairing onto the Solid phase for Antigen B; and Fig. 7 depicts a complex carrying Antigen C, which is captured via nucleotide complementary pairing onto the Solid phase for Antigen C.

Subsequently washing the complexes captured onto the individual solid phases, impurities not captured onto any of the phases, for example labeled substances and the like, can be removed. Figs. 8, 9 and 10 depict three types of the complexes captured on the independently and separately present solid phases after impurities are washed off; Fig. 8 depicts the complex carrying the Antigen A on the Solid phase for Antigen A, after washing; Fig. 9 depicts the complex carrying the Antigen B on the Solid phase for Antigen B, after washing; and Fig. 10 depicts the complex carrying the Antigen C on the Solid phase for Antigen C, after washing. The labels contained in the complexes captured on the individual solid phases are to be determined. For example, enzyme reactions are promoted in each independent reaction system to determine the level of the label via individual colors and the like. When the labels are fluorescence substances, dyes, metal colloids or the like, assay can be done without such enzyme reactions.

As described above, the method for assaying one or more species of antigens exemplifies and explains the case where three types of antigens are to be assayed. In accordance with the present invention, reacting a testing sample with one or more species of antibodies provided with different nucleotide properties from nucleotides of different sequences, each antibody being capable of binding one of subjective substances, together with labeled antibodies each recognizing one of the subjective substances, thereby forming immune complexes, and reacting the nucleotides bound to the antibodies with solid phases bound with groups of nucleotides (nucleotides-bound solid phases), each of the groups having a partially or entirely complementary sequence to that of the nucleotides bound to the antibody, to capture the immune complexes onto the solid phases, subsequently washing the solid phases bound with the immune complexes, assaying or detecting thereafter the level of the labels in the immune complexes, one or more species of subjective substances can be assayed or detected in the testing sample.

The above example illustrates a method for assaying antigens in a testing sample, but the present invention also encompasses the method for assaying antibodies in a testing sample. With reference to Figs. 11 to 19, explanation will follow about the method for assaying one or more species of antibodies as the immunological ligands to be assayed. Fig. 11 schematically depicts the assay reagent for assaying one or more species of antibodies, i.e. Antibodies A, B and C.

Fig. 12 illustrates one example of the nucleotides-bound solid phase to be used in combination with the assay reagent for assaying one or more species of antibodies in accordance with the present invention; Fig. 12(a) depicts a solid phase bound with nucleotides having a complementary base sequence to that of Nucleotide ON1 (Solid phase for

Antibody A); Fig. 12(b) depicts a solid phase bound with nucleotides having a complementary base sequence to that of Nucleotide ON2 (Solid phase for Antibody B); and Fig. 12(c) depicts a solid phase bound with nucleotides having a complementary base sequence to that of Nucleotide ON3 (Solid phase for Antibody C). These different types of solid phases are independently and separately present. When used in combination with the assay reagent for assaying one or more species of antibodies of Fig. 11, these individual solid phases constitute one example of the assay kit for assaying one or more species of antibodies in accordance with the present invention.

When the assay reagent for assaying one or more species of antibodies as depicted in Fig. 11 is added to a testing sample containing Antibodies A, B and C, complexes of (antigen-nucleotides conjugate)-antibody-labeled substance are formed, individually for Antibodies A, B and C, in a single reaction solution. Fig. 13 depicts the stage.

Then, the reaction solution containing the complexes of (antigen-nucleotides conjugate)-antibody-labeled substance is brought into contact with the independently and separately present Solid phases for Antibodies A, B and C, to prepare complementary pairs through hybridization between the nucleotides contained in the complexes and the nucleotides of the Solid phases of Antibodies A, B and C, having complementary base sequences to those of the nucleotides in the complexes.

Figs. 14, 15 and 16 depict the complexes captured via hybridization onto the individual solid phases; Fig. 14 depicts the complex carrying Antibody A, captured via the nucleotide complementary pairing onto the Solid phase for Antibody A; Fig. 15 depicts the complex carrying Antibody B, captured via the nucleotide complementary pairing onto the Solid phase for Antibody B; and Fig. 16 depicts the complex carrying Antibody C, captured via the nucleotide complementary pairing onto the Solid phase for Antibody C.

Subsequently washing the complexes captured onto the individual solid phases, impurities not attached onto the solid phases, for example, labeled substances, can be removed. In such manner, complexes captured on the individual three types of solid phases and from which impurities are removed, i.e. the complex carrying Antibody A, captured onto the Solid phase for Antibody A, as shown in Fig. 17, the complex carrying Antibody B, captured onto the Solid phase for Antibody B as shown in Fig. 18 and the complex carrying Antibody C, captured onto the Solid phase for Antibody C, as shown in Fig. 19, can be recovered individually.

The labels contained in the complexes captured onto the individual solid phases should be determined. For example, enzyme reactions and the like should be carried out in independent reaction systems, to determine the levels of the labels via color tones. When the labels are fluorescence substances, dyes and metal colloids and the like, assay can be done without such enzyme reaction.

The solid phase-nucleotides conjugate as the Solid phase C, for use in the assay reagent for assaying one or more species of immunological ligands in accordance with the present invention, is stable in dry states, while the conjugate can be stored in stability even in solution in the presence of EDTA. Thus, the conjugate can be kept stable in the state of the reagent.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 depicts the scheme of the general process of conventional immunoassay methods using labeled compounds;
Fig. 2 depicts one example of the assay reagent for assaying one or more species of antigens, schematically depicting the assay reagent for assaying one or more species of antigens, Antigens A, B and C;
Fig. 3 depicts one example of the solid phase bound with a nucleotide to be used in combination with the assay reagent for as saying one or more species of antigens in accordance with the present invention;
Fig. 4 depicts how the complex of (antibody-nucleotides conjugate)-antigen-labeled substance can be formed for one or more species of antigens contained in a single reaction solution in accordance with the present invention;
Fig. 5 depicts the state wherein a complex carrying Antigen A is captured through the complementary pairing of nucleotides onto the Solid phase for Antigen A in accordance with the present invention;
Fig. 6 depicts the state wherein a complex carrying Antigen B is captured through the complementary pairing of nucleotides onto the Solid phase for Antigen B in accordance with the present invention;
Fig. 7 depicts the state wherein a complex carrying Antigen C is captured through the complementary pairing of nucleotides onto the Solid phase for Antigen C in accordance with the present invention;
Fig. 8 depicts the state of the complex carrying Antigen A, complementarily paired onto the Solid phase for Antigen A, after washing;
Fig. 9 depicts the state of the complex carrying Antigen B, complementarily paired onto the solid phase for Antigen B, after washing;
Fig. 10 depicts the state of the complex carrying Antigen C, complementarily paired onto the Solid phase for Antigen C, after washing;
Fig. 11 depicts one example of the assay reagent for assaying one or more species of antibodies, schematically depicting the assay reagent for assaying one or more species of antibodies, Antibodies A, B and C;
Fig. 12 depicts one example of the solid phase bound with nucleotides to be used in combination with the assay reagent for assaying one or more species of antibodies in accordance with the present invention;

Fig. 13 depicts how the complex of (antigen-nucleotides conjugate)-antibody-labeled substance can be formed, individually for Antibodies A, B and C, in a single reaction solution in accordance with the present invention;

Fig. 14 depicts the state wherein a complex carrying Antibody A is captured through the complementary pairing of nucleotides onto the Solid phase for Antibody A in accordance with the present invention;

Fig. 15 depicts the state wherein a complex carrying Antibody B is captured through the complementary pairing of nucleotides onto the Solid phase for Antibody B in accordance with the present invention;

Fig. 16 depicts the state wherein a complex carrying Antibody C is captured through the complementary pairing of nucleotides onto the Solid phase for Antibody C in accordance with the present invention;

Fig. 17 depicts the state of the complex carrying Antibody A, complementarily paired onto the Solid phase for Antibody A, after washing;

Fig. 18 depicts the state of the complex carrying Antibody B, complementarily paired onto the solid phase for Antibody B, after washing;

Fig. 19 depicts the state of the complex carrying Antibody C, complementarily paired onto the solid phase for Antibody C, after washing;

Fig. 20 depicts the absorbance of the labeled substances captured on the solid phases A, B and C, by the method for assaying one or more species of immunological ligands in Example 1; and

Fig. 21 depicts the absorbance of the labeled substances captured on the solid phases A, B and C, by the method for assaying one or more species of immunological ligands in Example 2.

BEST MODE FOR CARRYING OUT THE INVENTION

The present invention will now be explained in details in examples.

[Example 1]

The following three types of oligonucleotides with an amino group at 5' terminus were synthesized by using an automatic DNA synthesizer, Type 391A, manufactured by Applied Biosystems:

Amino group-GAA TTC CCG GGG ATC CGT CG (referred to as "Nucleotide Pair 1(+)");

Amino group-GCC AAG CTT GGC TGC AGG TC (referred to as "Nucleotide Pair 2(+)");

Amino group-AAG CTT GCA TGC CTG CAG GT (referred to as "Nucleotide Pair 3(+)").

Using glutaraldehyde, these oligonucleotides were individually covalently bonded to polystyrene beads with an amino group introduced therein, which were then stored in 10 mM sodium phosphate buffer and 0.1 M sodium chloride, pH 7.0, containing 0.1 % skimmed milk, 0.1 % sodium azide and 5 mM EDTA (ethylene diamine tetraacetic acid). (Hereinafter, the solid phases individually bound with Nucleotide pairs 1(+), 2(+) and 3(+) are referred to as Solid Phases A, B and C, respectively.)

Rabbits were immunized individually with antigens, namely Cholera toxin (CT) generated from Vibrio cholerae, thermo-stable direct haemolysin (TDH) generated from Vibrio parahaemolyticus and Campylobacter jejuni, to prepare antibodies against the individual antigens. From each of the individual antibodies, the F(ab')2 was prepared from the IgG to prepare the Fab', according to the method of Y. Oku, et al., Microbiol. Immunol., 32, pp. 807-816, 1988. The Nucleotide pair 1(-) having a base sequence complementary to that of the Nucleotide pair 1(+) was covalently bound to the anti-CT-Fab'. Similarly, the Nucleotide pair 2(-) having a base sequence complementary to that of the Nucleotide pair 2(+) was covalently bound to the anti-TDH-Fab'. Furthermore, the Nucleotide pair 3(-) having a base sequence complementary to that of the Nucleotide pair 3(+) was also covalently bound to the anti-CJ-Fab'. According to the method of Y. Oku, et al., Microbiol. Immunol., 32, pp. 807-816, 1988, horse radish peroxidase (HRPO) was introduced into each of the Fab's at the SH group of their hinge parts.

Using 10 mM Bicine buffer, 0.3 M sodium chloride, 0.1 % bovine serum albumin, 0.002 % thimerosal, and 5 mM EDTA, pH 8.3, a mixture solution containing six types of the following complexes was prepared; 20 pmol/ml Nucleotide pair 1(-) bound-anti-CT-Fab', 20 pmol/ml Nucleotide pair 2(-) bound-anti-TDH-Fab', 20 pmol/ml Nucleotide pair 3(-) bound-anti-CJ-Fab', 800 ng/ml HRPO bound-anti-CJ-Fab', 800 ng/ml HRPO bound-anti-TDH-Fab', and 1600 ng/ml HRPO bound-anti-CJ-Fab'.

1.5 ml of the solution was individually put into three test tubes. Sample 1 (1.5 ml) containing 10 ng/ml CT was added into one of the tubes; Sample 2 (1.5 ml) containing 10 ng/ml TDH was added to another tube; and Sample 3 (1.5 ml) containing CJ at a 0.005 turbidity at 600 nm was added to the remaining tube. Then, these tubes were subjected to reaction at 37° C for one hour.

The solution in each of the tubes was divided by a 0.5-ml portion into six tubes for subsequent reaction at 37° C for one hour; Solid phase A was added into two of the tubes; Solid phase B was added to other two tubes; and Solid phase C was added into the remaining two tubes. After discarding the reaction solution, then, the remaining solid phases were washed in 0.3 M sodium chloride solution (5 ml x 3 times). After washing, the individual solid phases were independently transferred into other test tubes. The HRPO attached onto the individual solid phases was assayed according to the

method of Y. Oku, et al., Microbiol. Immunol., 32, pp. 807-816, 1988. The results are shown in Table 1 and Fig. 20.

[Table 1]

Sample name	For assaying CT	For TDH	For CJ	Results
Sample 1	2.652	0.061	0.039	CT
Sample 2	0.03	0.915	0.053	TDH
Sample 3	0.068	0.078	0.357	C.jejuni

As apparently shown in Table 1 and the graphs of Fig. 20, Sample 1 containing CT significantly reacts with the Solid phase A for assaying CT; Sample 3 containing CJ significantly reacts with the Solid phase C for assaying CJ. It has been indicated that even a single reagent can assay one or more species of subjective substances, using the substances and method described in the present Example.

[Example 2]

Process 1:

The following four types of oligonucleotides with an amino group at 5' terminus were synthesized by using an automatic DNA synthesizer, Type 391A, manufactured by Applied Biosystems:

Amino group-GAA TTC CCG GGG ATC CGT CG (referred to as "Nucleotide Pair 1(+)");
Amino group-AAG CTT GCA TGC CTG CAG GT (referred to as "Nucleotide Pair 3(+)");
Amino group-GGC GAC TGT CGA ACC GGA AA (referred to as "Nucleotide Pair 5(+)"); and
Amino group-CCA CCC CTA CTC CTA ATC CC (referred to as "Nucleotide Pair 6(+)).

Using glutaraldehyde, these oligonucleotides were individually covalently bonded to polystyrene beads with an amino group introduced therein, which were then stored in 10 mM sodium phosphate buffer and 0.1 M sodium chloride, pH 7.0, containing 0.1 % gelatin, 0.002 % thimerosal and 5 mM EDTA (ethylene diamine tetraacetic acid).

Process 2:

A sulfhydryl group was preliminarily introduced into various types of allergens. The allergens, i.e. wheat flour, egg white, soy bean and rice for clinical tests, purchased from Torii Pharmaceutical Kabushiki Kaisha, were concentrated under cooling in ice by means of a YM-2 ultrafiltration membrane. Subsequently, these allergens were dialyzed against 0.1 M sodium phosphate buffer, pH 7.0. After dialysis, an excess amount of N-succinimidyl-S-acetylthioacetate (SATA; manufactured by Pierce, Co. Ltd.) reacted with the resulting allergens at 37 ° C for one hour. After the completion of the reaction, 1 M Tris-HCl buffer, pH 7.0 and 1 M hydroxyamine, pH 7.0 were independently added to the individual allergen reaction products to final concentrations of 0.1 M, respectively, for reaction at 37° C for 15 minutes to promote deprotection. After the termination of the reaction, the reaction products were then applied to a gel filtration support, Sephadex G-25, equilibrated with 0.1 M sodium phosphate buffer, pH 6.0 containing 5 mM EDTA, to collect fractions corresponding to protein. The fractions were concentrated against the YM-2 ultrafiltration membrane, to recover four types of concentrated allergens introduced with a sulfhydryl group.

Process 3:

By the same method in the same manner, oligonucleotides having sequences complementary to those of the oligonucleotides synthesized in the Process 2 were synthesized, to recover four types of oligonucleotides each with an amino group at 5' terminus. An oligonucleotide complementary to the Nucleotide pair 1(+) was defined as Nucleotide pair 1(-); other complementary oligonucleotides were designated as Nucleotide pair 3(-), Nucleotide pair 5(-) and Nucleotide pair 6(-).

Process 4:

An excess amount of N-(ε-maleimide caproyloxy) succinimide (abbreviation: EMCS) reacted with the four types of the complementary oligonucleotides produced in the Process 3 at 37° C for one hour, to introduce the maleimide group into the 5' termini of the individual oligonucleotides. After the completion of the reaction, the four types of the oligonucleotides introduced with a maleimide group were purified through ethanol precipitation, according to a routine method.

Process 5:

The four types of the concentrated allergens introduced with a sulfhydryl group as prepared in the Process 3, were mixed with the four types of the oligonucleotides introduced with a maleimide group as prepared in the Process 4, for reaction at 37° C for one hour, to produce an allergen mixture introduced with the oligonucleotides. The wheat flour allergen was bound to the Nucleotide pair 1(-); the soy bean allergen was bound to Nucleotide pair 3(-); the egg white allergen was bound to Nucleotide pair 5(-); and the rice allergen was bound to Nucleotide pair 6(-).

Process 6:

The allergen mixture bound with the four types of the oligonucleotides, which was prepared in the Process 5, was diluted with 10 mM sodium phosphate buffer, pH 7.0, containing 0.1 % gelatin, 0.3 M sodium chloride and 5 mM EDTA, to a final protein concentration of 1 µg/ml and a final concentration of anti-human IgE labeled with horse radish peroxidase to 100 ng/ml. The resulting diluted allergen mixture was defined as Reagent A. The Reagent A (7.2 ml) was poured into a test tube, followed by addition of patient serum (2.4 ml), for reaction at 37 ° C for one hour. The resulting reaction solution was defined as Reagent A mixture solution. The patient serum was collected independently from three patients.

Process 7:

After the termination of the reaction, Nucleotide pair 1(+)-bound solid phase, Nucleotide pair 3(+)-bound solid phase, Nucleotide pair 5(+)-bound solid phase and Nucleotide pair 6(+) -bound solid phase were individually divided into test tubes, followed by addition of the Reagent A mixture solution (400 µl) after the termination of the reaction, so as to react them together at 37° C for one hour.

Process 8:

After the reaction, the resulting solution was washed off three times in 0.3 M sodium chloride solution. Subsequently, the individual solid phases were transferred into fresh test tubes, where the activity of the enzyme attached onto the solid phases was assayed with 3, 3', 5, 5'-tetramethylbenzidine. Herein, the assay was carried out in duplicate.

The results are shown in bar graphs in Fig. 21. In the graphs of Fig. 21, the axis of abscissas represents the type of an allergen and the identity of patient serum for the individual allergens, while the axis of ordinates represents the absorbance at 450 nm.

Fig. 21 apparently shows that using the proteins introduced with oligonucleotides, the simultaneous detection of a plurality of allergen specific IgEs can be achieved.

INDUSTRIAL APPLICABILITY

In accordance with the present invention, the time required for the reaction of a mixture containing an immune complex-labeled substance with a nucleotides-bound solid phase is far shorter than the reaction time required for the antigen-antibody reaction utilizing the binding onto solid phase according to conventional methods. Thus, so-called non-specific binding of a labeled substance directly onto a solid phase can be decreased, thereby achieving a highly sensitive assay system.

In accordance with the present invention, a single reagent can detect or assay one or more species of immunological ligands, namely one or more species of antigens or one or more species of antibodies, so the time required for detecting or assaying them can be shortened rapidly.

Theoretically, the number of the combination of the base sequences between complementary nucleotides is almost infinite. Therefore, an almost infinite number of the detected combination of either one of such immunological pairs, namely one or more species of antigens or one or more species of antibodies, will be possible in accordance with the present invention.

Claims

1. An assay reagent for assaying one or more species of immunological ligands, characterized in that the reagent concurrently contains the following substance groups (A) and (B):

   Substance group (A): one or more species of immunological anti-ligand-nucleotides conjugates, in each of which nucleotides with a specific base sequence, independently selected depending on the species of an immunological ligand, are bound to an immunological anti-ligand having a specific immunological affinity to one of immunological ligands as different species of subjective substances; and

Substance group (B): labeled substances each having a specific affinity to one of the immunological ligands as the different species of subjective substances.

2. An assay kit for assaying one or more species of immunological ligands, comprising an assay reagent for assaying one or more species of immunological ligands which concurrently contains the following substance groups (A) and (B), and the following solid phase (C):

Substance group (A): one or more species of immunological anti-ligand-nucleotides conjugates, in each of which nucleotides with a specific base sequence, independently selected depending on the species of an immunological ligand, are bound to an immunological anti-ligand having a specific immunological affinity to one of immunological ligands as different species of subjective substances;

Substance group (B): labeled substances each having a specific affinity to one of the immunological ligands as the different species of subjective substances; and

Solid phase (C); solid phase-nucleotides conjugates wherein nucleotides having a base sequence complementarily binding to the nucleotides of the above Substance group (A) are immobilized onto a water-insoluble support.

3. An assay kit for assaying one or more species of immunological ligands according to claim 2, wherein the solid phase-nucleotides conjugate as the Solid phase (C) is produced by covalently binding the nucleotides at position 5' terminus or 3' terminus or an optional position other than the termini onto a water-insoluble support, directly or through a functional group inserted into the water-insoluble support.

4. An assay kit for assaying one or more species of immunological ligands according to claim 2, wherein the solid phase-nucleotides conjugate as the Solid phase (C) is produced by covalently binding the nucleotides at position 5' terminus or 3' terminus or an optional position other than the termini onto a bonding ligand, directly or through a functional group inserted into the bonding ligand to form a bonding ligand-nucleotides conjugate and by phisically absorbing said bonding ligand-nucleotides conjugate onto a water-insoluble support.

5. An assay kit for assaying one or more species of immunological ligands according to claim 4, wherein the bonding ligand is a protein.

6. An assay kit for assaying one or more species of immunological ligands according to claim 2, 3, 4 or 5, wherein the nucleotides have a base sequence partially or entirely capable of complementary binding.

7. An assay reagent or an assay kit for assaying one or more species of immunological ligands according to claim 1, 2, 3, 4, 5 or 6, wherein the label is an enzymatically active atomic group, biotin, avidin, digoxigenin, nucleotides, a metal colloid particle, a fluorescent substance, a luminescent substance, a metal compound, a ligand with a specific binding affinity or a radioisotope.

8. An assay reagent or an assay kit for assaying one or more species of immunological ligands according to claim 1, 2, 3, 4, 5, 6 or 7, wherein the nucleotides are an oligonucleotide or a polynucleotide.

9. An assay reagent or an assay kit for assaying one or more species of immunological ligands according to claim 1, 2, 3, 4, 5, 6, 7 or 8, wherein the immunological ligands are antibodies.

10. An assay kit for assaying one or more species of immunological ligands according to claim 1, 2, 3, 4, 5, 6, 7 or 8, wherein the immunological ligands are antigens.

11. A method for simultaneously assaying one or more species of immunological ligands, comprising:

(1) reacting together the Substance group (A) according to claim 2, the Substance group (B) according to claim 2, and a testing sample possibly containing one or more species of immunological ligands as subjective substances in a single reaction vessel, thereby forming complexes of (immunological anti-ligand-nucleotides conjugate)-immunological ligand-labeled substance depending on the type of each of the immunological ligands in the reaction solution;

(2) bringing the reaction solution possibly containing one or more species of such complexes of (immunological anti-ligand-nucleotides conjugate)-immunological ligand-labeled substance in contact with the independently and separately present Solid phases (C) according to claim 2, thereby forming complexes of (solid phase-nucleotides conjugate)-(immunological anti-ligand-nucleotides conjugate)-immunological ligand-labeled sub-

stance at the nucleotide regions thereof where one nucleotide region has a base sequence complementary to (a part or the whole of) the base sequence of the other nucleotide region; and

(3) detecting or assaying the label contained in the complexes formed in the above item 2.

12. A method for simultaneously assaying one or more species of immunological ligands according to claim 11, wherein the immunological ligands are antigens.

13. A method for simultaneously assaying one or more species of immunological ligands according to claim 11, wherein the immunological ligands are antibodies.

Fig. 1

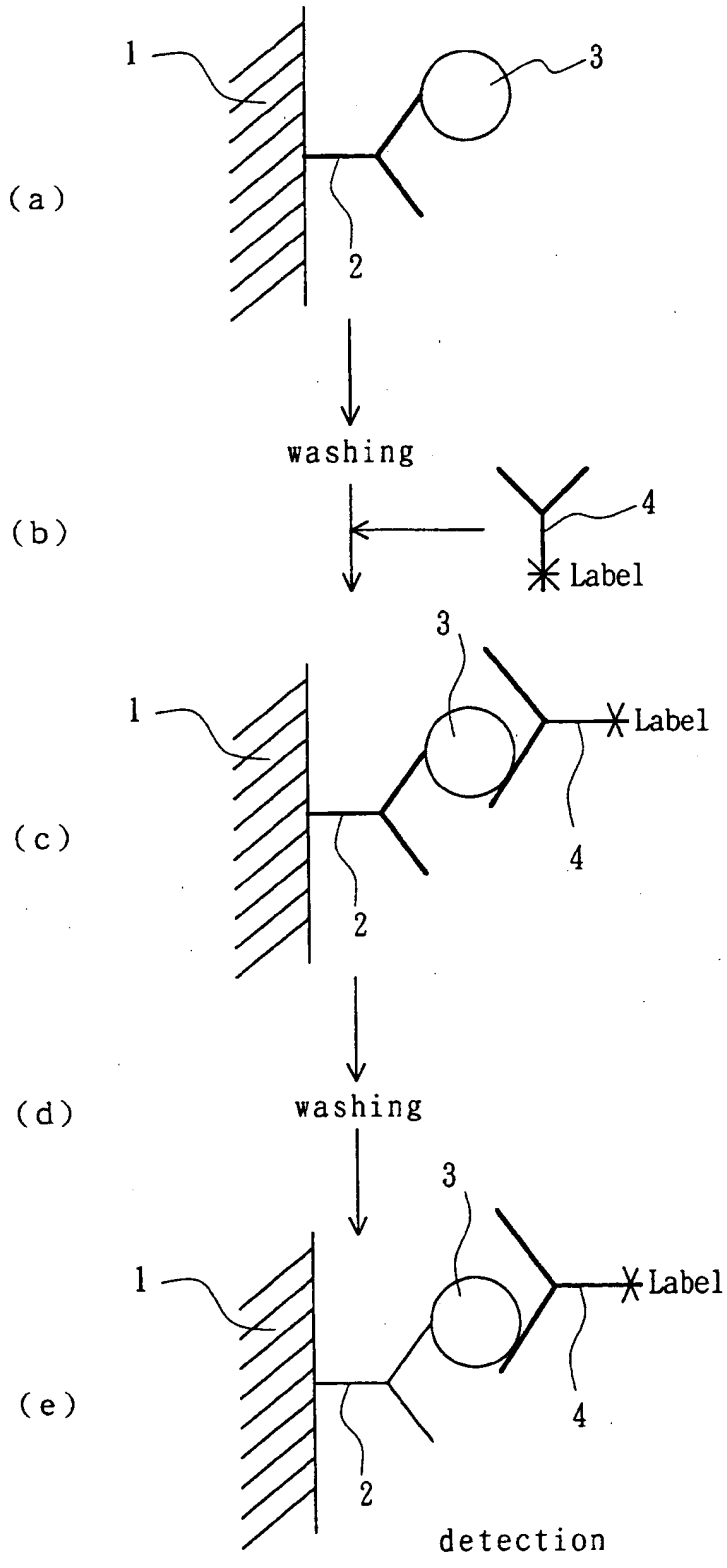
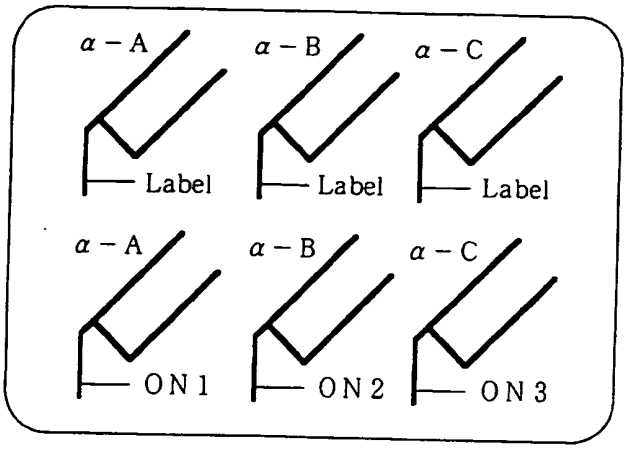

(a)
1
2
3
washing
(b)
4
Label
(c)
1
2
3
4
Label
washing
(d)
(e)
1
2
3
4
Label
detection

Fig. 2

Antigen assay reagent capable of assaying concurrently Antigen A,B,C



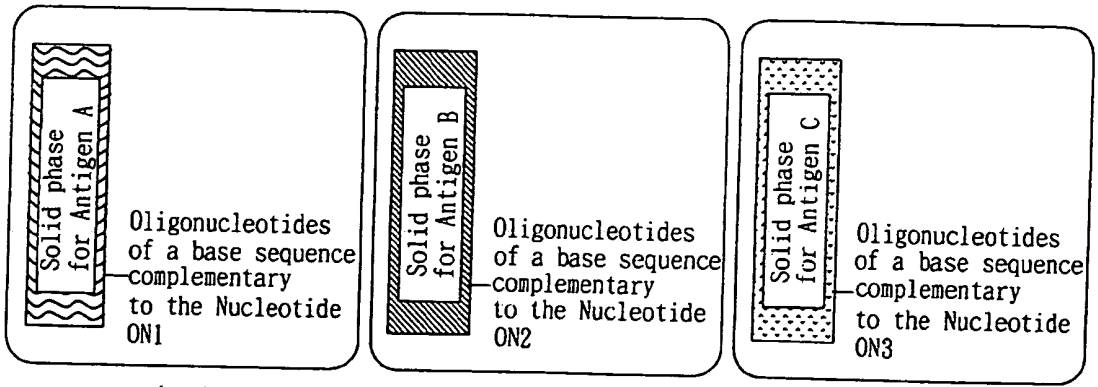
α − A, B, C : Antibodies against Antigen A,B,C, respectively

ON 1 : Oligonucleotide having a base sequence different from those of other Nucleotides ON2 and ON3

ON 2 : Oligonucleotide having a base sequence different from those of other Nucleotides ON1 and ON3

ON 3 : Oligonucleotide having a base sequence different from those of other Nucleotides ON1 and ON2

Fig. 3



(a) (b) (c)

Fig. 4

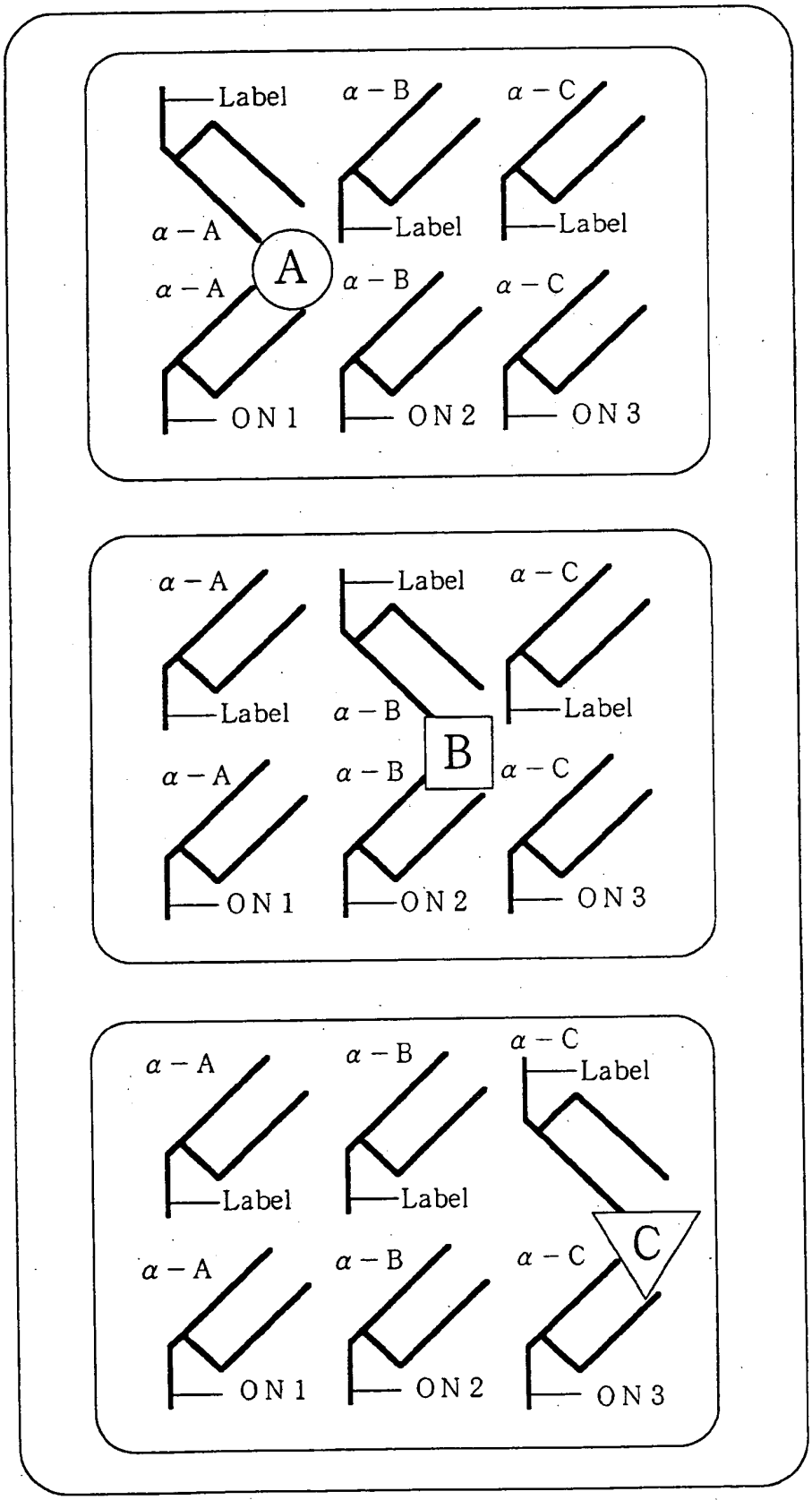

Label
α – B
α – C
α – A
A
α – A
α – B
α – C
ON 1
ON 2
ON 3
α – A
Label
α – C
α – B
B
α – A
α – B
α – C
ON 1
ON 2
ON 3
α – A
α – B
α – C
Label
Label
Label
C
α – A
α – B
α – C
ON 1
ON 2
ON 3

Fig. 5

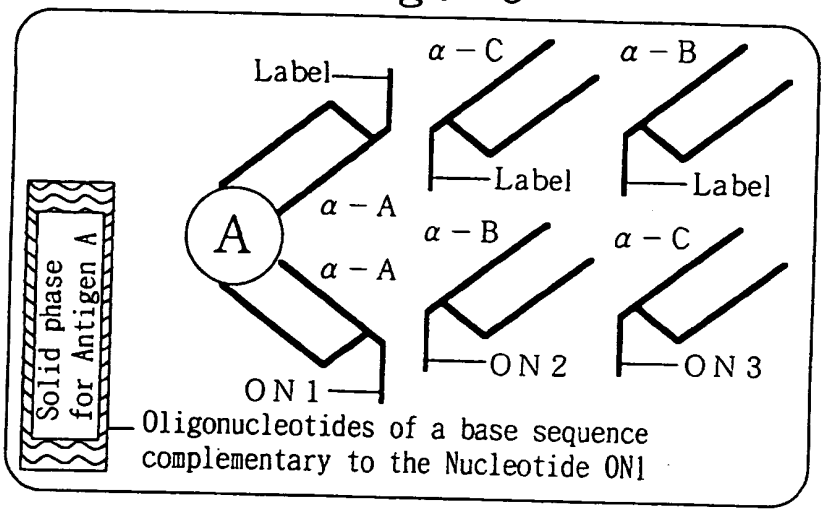


Fig. 6

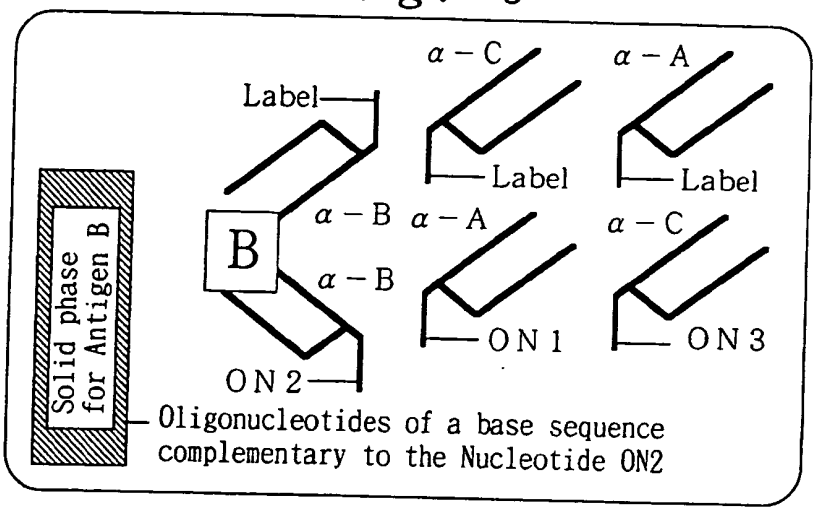


Fig. 7

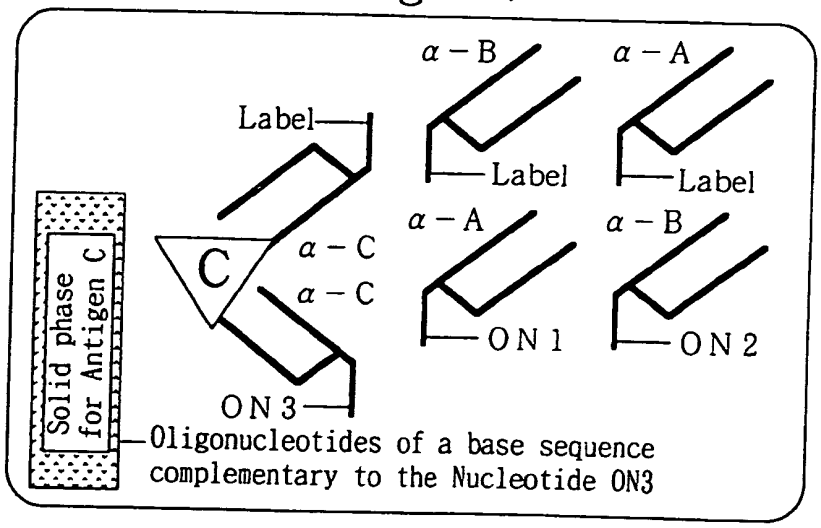


Fig. 8

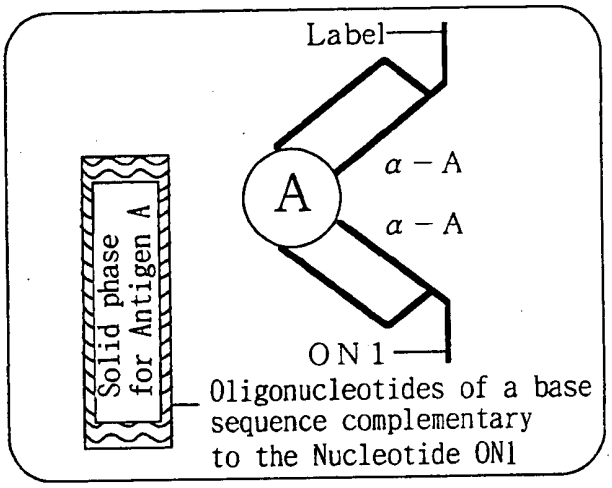


Fig. 9

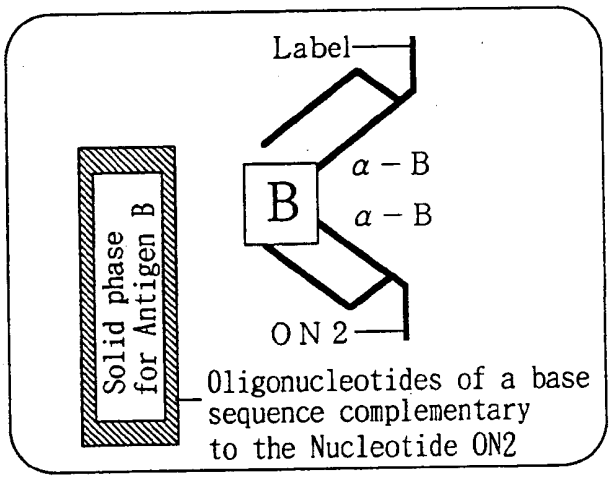


Fig. 10

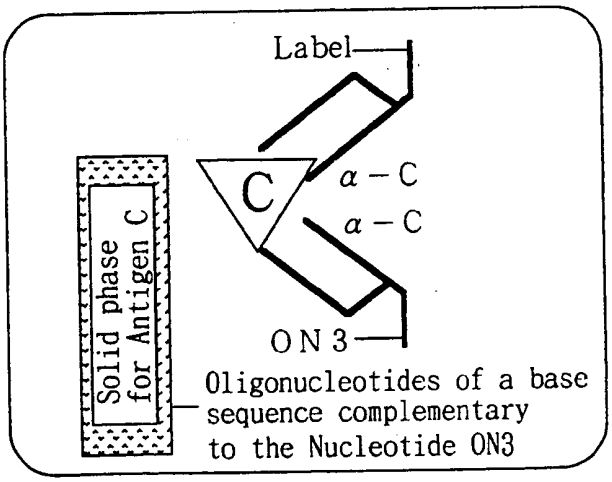
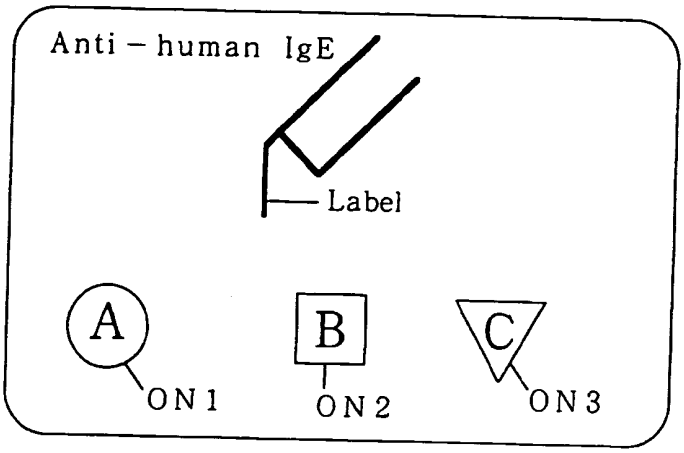


Fig. 11

Antibody assay reagent capable of assaying concurrently Antibody A,B,C



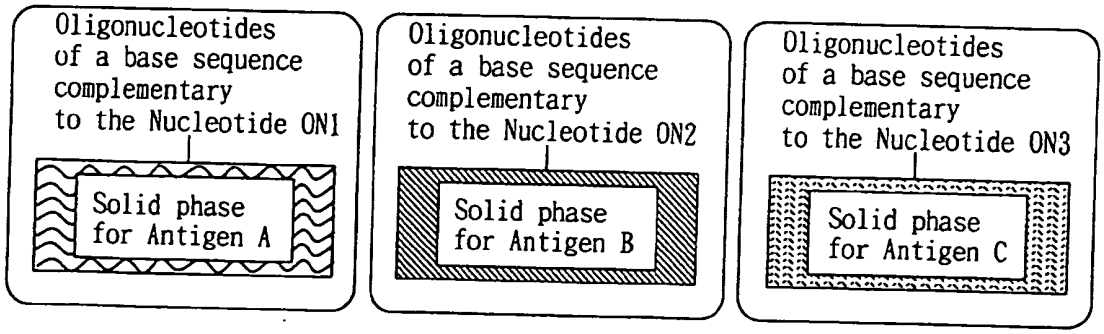
A, B, C : Antigen A,B,C, respectively

ON 1 : Oligonucleotide having a base sequence different from those of other Nucleotides ON2 and ON3

ON 2 : Oligonucleotide having a base sequence different from those of other Nucleotides ON1 and ON3

ON 3 : Oligonucleotide having a base sequence different from those of other Nucleotides ON1 and ON2

Fig. 12



(a) (b) (c)

Fig. 13

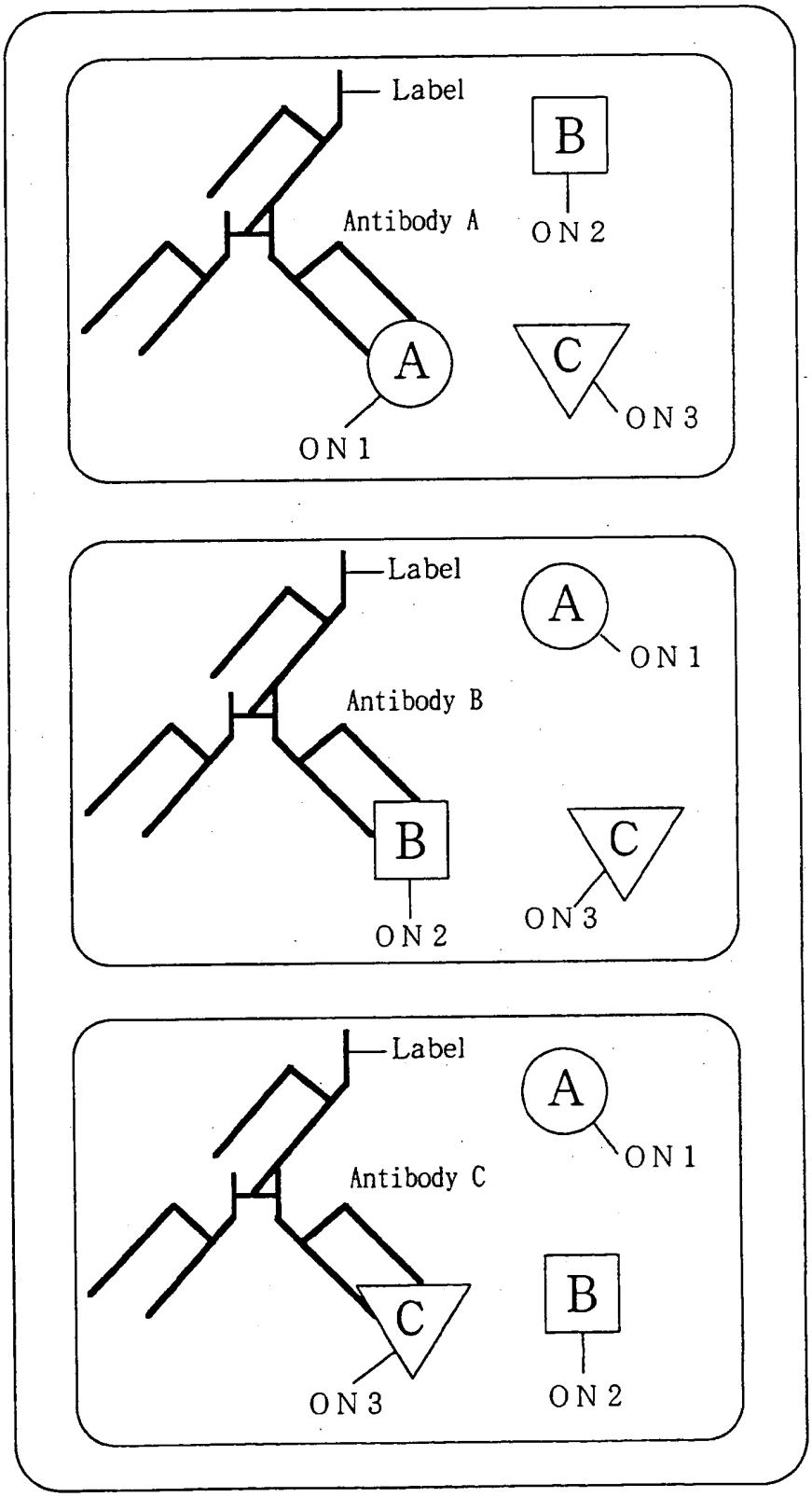

Label
B
Antibody A
ON 2
A
C
ON 3
ON 1
Label
A
ON 1
Antibody B
B
C
ON 2
ON 3
Label
A
ON 1
Antibody C
C
B
ON 3
ON 2

Fig. 14

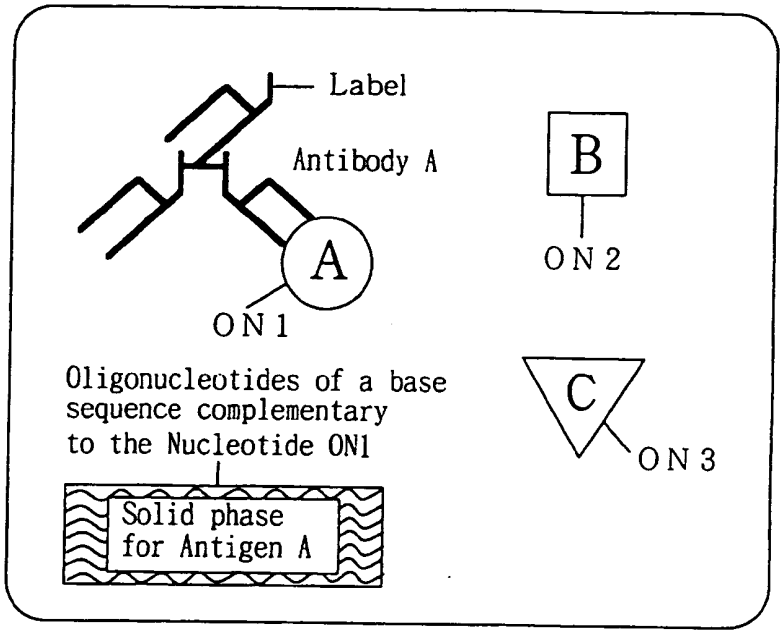

Label
Antibody A
A
ON1
B
ON2
C
ON3
Oligonucleotides of a base sequence complementary to the Nucleotide ON1
Solid phase for Antigen A

Fig. 15

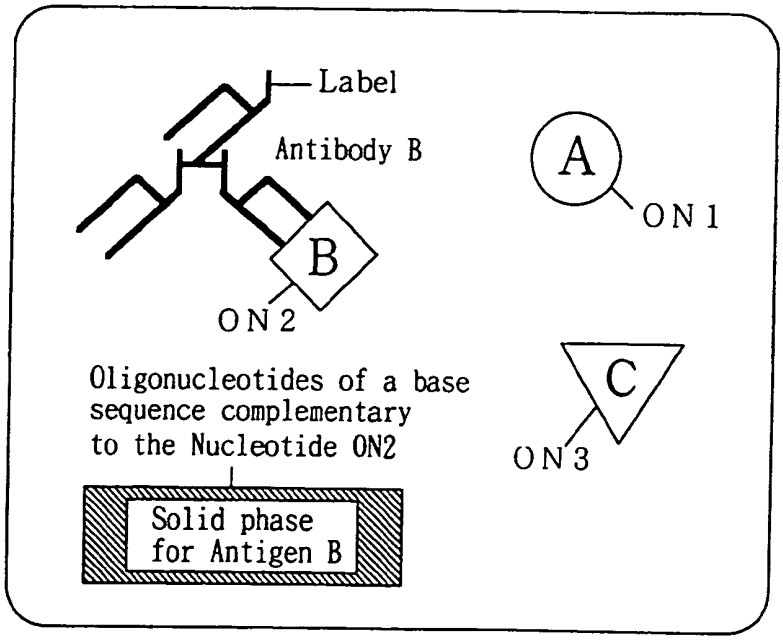

Label
Antibody B
B
ON2
A
ON1
C
ON3
Oligonucleotides of a base sequence complementary to the Nucleotide ON2
Solid phase for Antigen B

Fig. 16

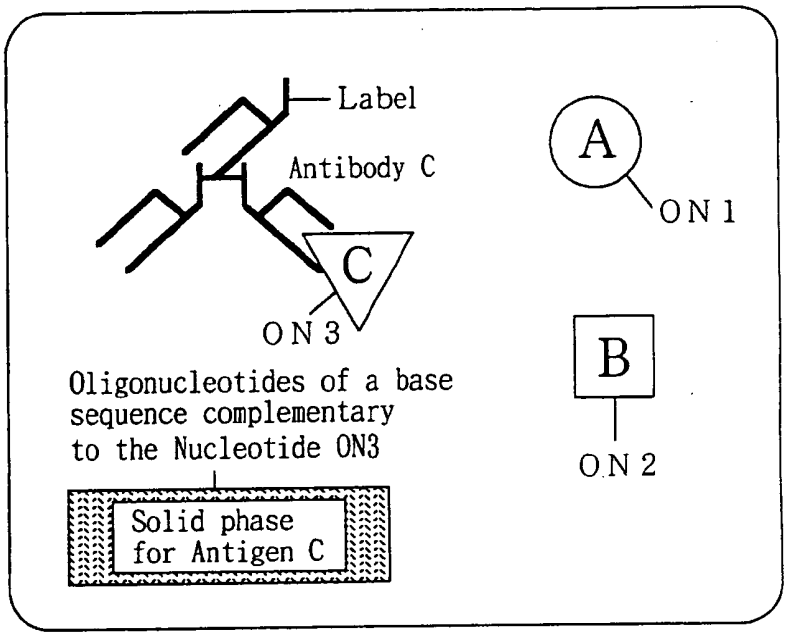

Label
Antibody C
C
ON 3
A
ON 1
B
ON 2
Oligonucleotides of a base sequence complementary to the Nucleotide ON3
Solid phase for Antigen C

Fig. 17

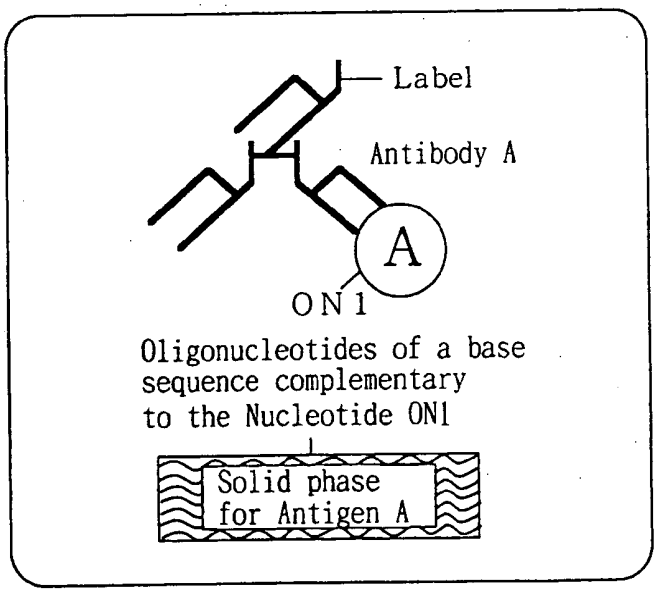

Label
Antibody A
A
ON 1
Oligonucleotides of a base sequence complementary to the Nucleotide ON1
Solid phase for Antigen A

Fig. 18

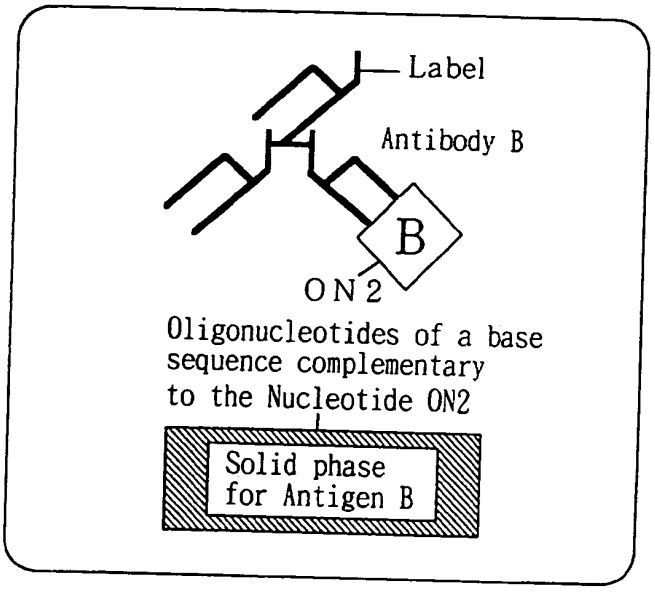

Label
Antibody B
B
ON2
Oligonucleotides of a base sequence complementary to the Nucleotide ON2
Solid phase for Antigen B

Fig. 19

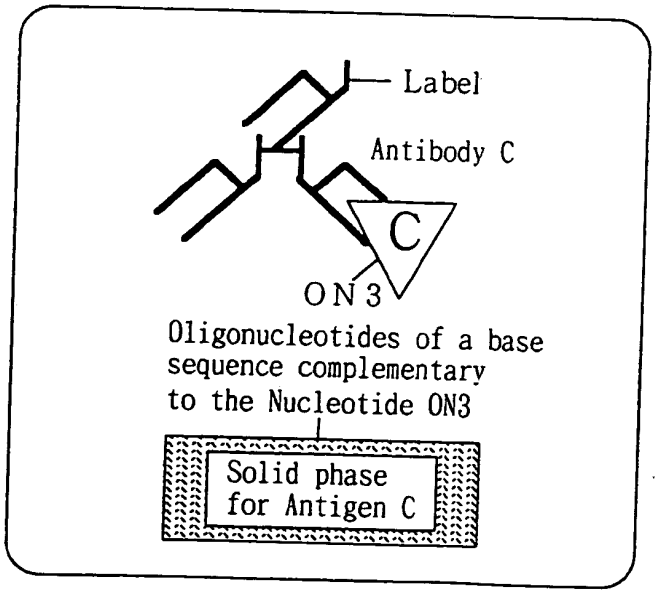

Label
Antibody C
C
ON3
Oligonucleotides of a base sequence complementary to the Nucleotide ON3
Solid phase for Antigen C

Fig. 20

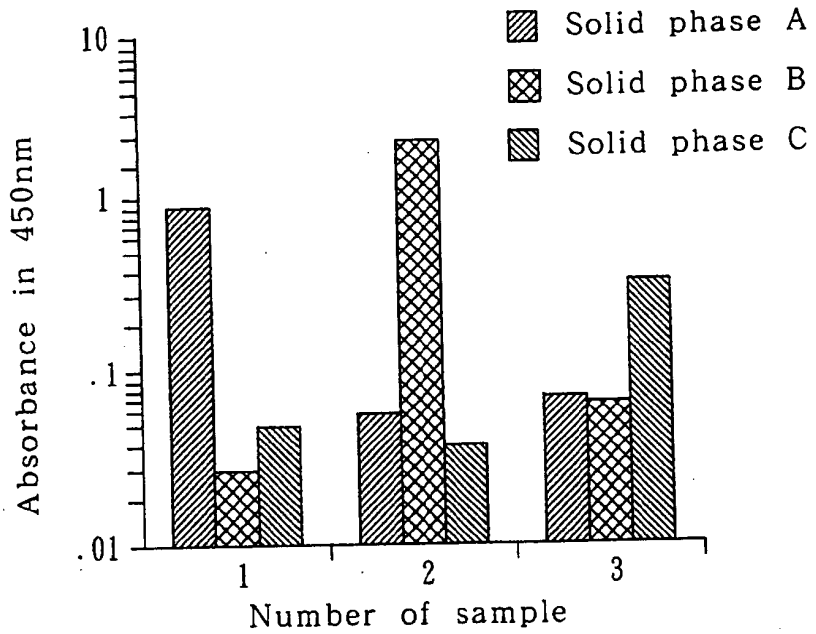
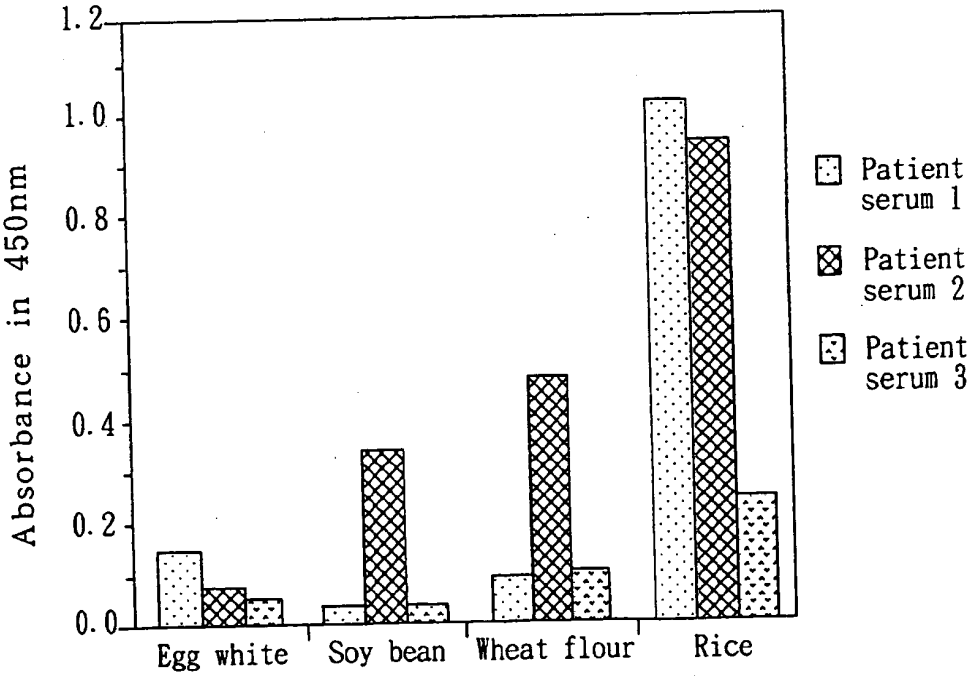

Solid phase A
Solid phase B
Solid phase C
Absorbance in 450nm
10
1
.1
.01
1
2
3
Number of sample

Fig. 21


Absorbance in 450nm
1.2
1.0
0.8
0.6
0.4
0.2
0.0
Egg white
Soy bean
Wheat flour
Rice
Patient serum 1
Patient serum 2
Patient serum 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/JP94/00725

A. CLASSIFICATION OF SUBJECT MATTER

Int. Cl^5 G01N33/543

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

Int. Cl^5 G01N33/543

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Jitsuyo Shinan Koho 1926 - 1994
Kokai Jitsuyo Shinan Koho 1971 - 1994

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	JP, A, 4-204379 (Hitachi, Ltd.), July 24, 1992 (24. 07. 92), Claim & EP, A, 488152	1-13
A	JP, A, 63-36151 (Showa Denko K.K.), February 16, 1988 (16. 02. 88), (Family: none)	1-13

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
June 6, 1994 (06. 06. 94)	June 28, 1994 (28. 06. 94)
Name and mailing address of the ISA/ Japanese Patent Office	Authorized officer
Facsimile No.	Telephone No.

Form PCT/ISA/210 (second sheet) (July 1992)